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(54) METHOD FOR AMPLIFYING ATP IN CHAINLIKE MANNER AND METHOD FOR TESTING TRACE ATP USING THE AMPLIFICATION METHOD

(57)Abstract:

PROBLEM TO BE SOLVED: To provide a new method for amplifying ATP in a chainlike manner, and a method for testing trace ATP by attaining sensitivity and accuracy of the measurement for bioluminescence using the method.

SOLUTION: The first reaction which converts ATP to 2 molecules of ADP by reacting the ADP with an adenylate kinase in the presence of trace ATP and the second reaction which converts the 2 molecules of ADP to 2 molecules of ATP and polyphosphoric acid compound by reacting the 2 molecules of ADP with a polyphosphoric acid kinase in the presence of polyphosphoric acid compound are paired to form a reaction system. The paired reaction system is repeated plural times, and thereby, ATP is amplified at a factor of the power of 2 according to the number of the repetitions of the reaction.

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CLAIMS

[Claim(s)]

[Claim 1] Under the 1st reaction make AMP react with the adenylate kinase and dyad ADP is made to change into the bottom of existence of ATP of a minute amount, and existence of a polyphosphoric acid compound The 2nd reaction which this dyad ADP is made to react with a polyphosphoric acid kinase, and is made to change into dyad ATP and a polyphosphoric acid compound by repeating the system of reaction of a couple, and the system of reaction of nothing and this couple two or more times, and making them react How to make ATP characterized by making ATP amplify by the exponentiation of 2 according to the count of a reaction amplify continuously.

[Claim 2] Said polyphosphoric acid compound is the approach of being the polyphosphoric acid compound generated by chemosynthesis and making ATP according to claim 1 which comes to use that in which at least 10-100 phosphoric acids carried out the polymerization to the shape of a straight chain amplifying continuously.

[Claim 3] Said polyphosphoric acid compound is the approach of being the polyphosphoric acid compound of the bacteria origin and making ATP according to claim 1 which comes to use that in which at least 10-1000 phosphoric acids carried out the polymerization to the shape of a straight chain amplifying continuously.

[Claim 4] Said polyphosphoric acid compound is the approach of making ATP according to claim 3 which it comes to biosynthesize from ATP amplifying continuously by the catalysis of polyphosphoric acid synthetic enzyme.

[Claim 5] How to inspect ATP of ultralow volume by measuring the amount of luminescence which was made to amplify ATP of ultralow volume by the approach of making either of claim 1 to claims 4 amplifying ATP of a publication continuously, was made to react with luciferase under existence of luciferin and dissolved oxygen, was made to generate AMP and luminescence, and was generated.

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DETAILED DESCRIPTION

[Detailed Description of the Invention]

[0001]

[The technical field to which invention belongs] It is an approach to which the amount of ATP generate in the metabolic turnover and the biosynthesis system of animals and plants is made to increase continuously, is detect a bioluminescence using this approach, and since it can detect ATP of the ultralow volume which was not able to be detected conventionally, this invention detects the microorganism which is not visible at food works etc., and can inspect cleanliness or it can apply it to measure the freshness of food, such as meat, a fresh fish, and vegetables.

[0002]

[Description of the Prior Art] ATP is the index of the live living thing. Therefore, health inspection of the microorganism which made light the index is conducted by presenting a bioluminescence with ATP of the microorganism origin. (For example, JP,6-34757,B, registration No. 1911659) However, in the case of the microorganism of a minute amount, the bioluminescence originating in ATP is not fully performed.

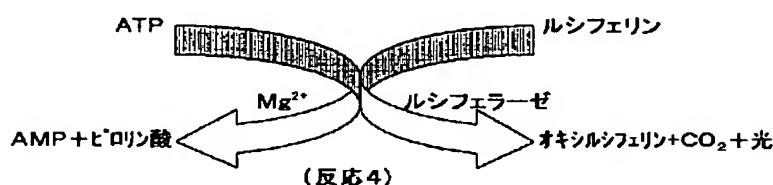
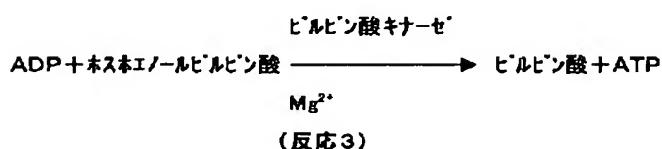
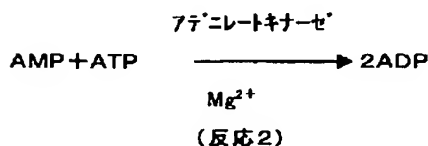
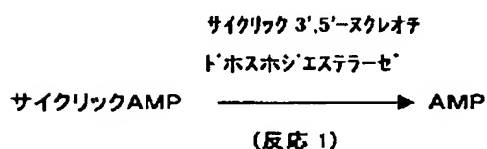
[0003] So, the technique characterized by what "a bioluminescence reaction is performed for in the approach of measuring the bioluminescence generated by luciferase under coexistence of a polyphosphoric acid compound or its salt, and a sulfhydryl compound" is indicated by JP,8-47399,A. Thereby, the new effectiveness of enhancement of a bioluminescence can be acquired and it is effective in measurement of a bioluminescence reaction being possible and a diffusion rate increasing by the activity of common RUMINO meter.

[0004] However, the approach indicated by above-mentioned JP,8-47399,A is the technique of stability of a bioluminescence, and it has the fault which luminescence decreases with time as not the technique to which the amount of ATP is made to increase but ATP is consumed.

[0005] Moreover, the assay of cyclic AMP by the bioluminescence method shown by the following reaction formulae is indicated by JP,9-234099,A.

[Formula 1]

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[0006] this approach -- cyclic AMP -- cyclic 3' and 5' -- it hydrolyzes by - nucleotide phosphodiesterase and AMP is generated to the system of reaction -- making (reaction 1) -- under existence of ATP of magnesium ion and a minute amount, this AMP is made to react with the adenylate kinase, and it changes into ADP -- making (reaction 2) -- under existence of magnesium ion and phosphoenolpyruvic acid this ADP is made to react with a pyruvate kinase, and it changes into ATP and a pyruvic acid -- making (reaction 3) -- under existence of luciferin, magnesium ion (or other metal ions), and dissolved oxygen ATP is made to react with luciferase and luminescence is generated -- making (reaction 4) -- it is characterized by the approach (METHODS IN ENZYMOLOGY 38, 62-65; 1974) of carrying out the quantum of cyclic AMP by measuring the amount of luminescence generated at (the reaction 4).

[0007] However, although this approach changes into ADP AMP changed from cyclic AMP and recycle of ATP is further performed by the reaction of a pyruvate kinase as ATP under existence of phosphoenolpyruvic acid in this ADP, the amount of ATP is not necessarily increasing.

[0008]

[Problem(s) to be Solved by the Invention] The above-mentioned conventional technique is detecting a bioluminescence using this approach, while inventing the approach to which the amount of ATP is made to increase continuously in a completely different viewpoint, and this invention makes it a technical problem to offer the approach of detecting ATP of the ultralow volume which was not able to be detected conventionally.

[0009]

[Means for Solving the Problem] In order to solve the above-mentioned technical problem, invention of claim 1 under the 1st reaction make AMP react with the adenylate kinase and dyad ADP is made to change into the bottom of existence of ATP of a minute amount, and existence of a polyphosphoric acid compound The 2nd reaction which this dyad ADP is made to react with a polyphosphoric acid kinase,

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and is made to change into dyad ATP and a polyphosphoric acid compound by repeating the system of reaction of a couple, and the system of reaction of nothing and this couple two or more times, and making them react. The technical means of making ATP amplify by the exponentiation of 2 according to the count of a reaction were provided.

[0010] In the 1st reaction of the system of reaction, ATP and AMP react promptly and are changed into dyad ADP by the adenylate kinase (the 1st reaction). Subsequently, by the polyphosphoric acid kinase, this dyad ADP reacts with polyphosphoric acid, and is changed into dyad ATP and polyphosphoric acid (the 2nd reaction). And although it moves to the 2nd reaction of the system of reaction, at this time, said dyad ATP produced in the 1st system of reaction reacts with dyad AMP, and is changed into ADP of four molecules (the 1st reaction). Subsequently, by the polyphosphoric acid kinase, ADP of these four molecules reacts with polyphosphoric acid, and is changed into ATP of four molecules, and polyphosphoric acid (the 2nd reaction). Hereafter, ATP increases by being repeated two or more times with the 3rd time of the system of reaction, the 4th time of the system of reaction, and 5th time -- of the system of reaction. ATP of the minute amount added to the 1st time of the system of reaction becomes a trigger, and henceforth, it happens continuously, as long as AMP and polyphosphoric acid exist, it will continue for a long time, and according to the count of a reaction of the system of reaction, ATP will increase this reaction by the exponentiation of 2.

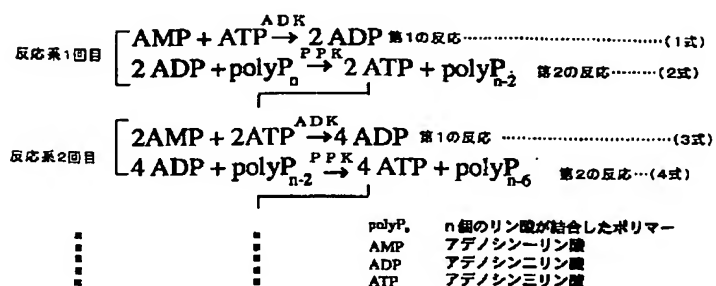
[0011] Moreover, said polyphosphoric acid compound is a polyphosphoric acid compound generated by chemosynthesis, and is good to use that in which at least 10-100 phosphoric acids carried out the polymerization to the shape of a straight chain. A polyphosphoric acid compound (PolyP_n) is that with which n phosphoric acids were connected, and in invention of claim 2, since at least 10-100 phosphoric acids (MI3P) are connected in 1 molecule of a polyphosphoric acid compound, conversion to ATP from ADP in the 2nd reaction of claim 1 is performed easily, and makeup of the phosphoric acid at the time of reproducing ATP becomes little, and becomes economical. Moreover, the reaction time of the system of reaction continues with many phosphoric acids. [0012] Furthermore, said polyphosphoric acid compound is a polyphosphoric acid compound of the bacteria origin, and is good to use that in which at least 10-1000 phosphoric acids carried out the polymerization to the shape of a straight chain. In invention of claim 3, since at least 10-1000 phosphoric acids (MI3P) are connected in 1 molecule of a polyphosphoric acid compound, it is carried out still more easily, and makeup of a phosphoric acid becomes little and the conversion to ATP from ADP becomes economical. Moreover, the reaction time of the system of reaction carries out long duration continuation with many phosphoric acids.

[0013] And if said polyphosphoric acid compound is biosynthesized from ATP by the catalysis of polyphosphoric acid synthetic enzyme, it will improve the yield of a polyphosphoric acid compound and will become possible [generating a polyphosphoric acid compound cheaply]. [0014] If the amount of luminescence which was made to amplify ATP of ultralow volume by the approach of making ATP amplifying continuously, was made to react with luciferase under existence of luciferin and dissolved oxygen, was made to generate AMP and luminescence, and was generated is measured, since the quantity of light equivalent to ATP of the part which increased will be obtained, ATP of the ultralow volume which was not able to be detected conventionally can be detected. Theoretically, ATP of the monad which exists in the first system of reaction is also detectable. [0015]

[Embodiment of the Invention] The gestalt of operation of this invention is explained. The theoretical reaction formula of this invention is shown below.

[Formula 2]

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[0016] The adenylate kinase (ADK) used as the catalyst of a reaction is an enzyme which produces dyad adenosine diphosphate (ADP), when adenosine monophosphate (AMP) and an adenosine triphosphate (ATP) are made to react. Moreover, a polyphosphoric acid kinase (PPK) is an enzyme which ADP and polyphosphoric acid (PolyP) are made to react and is changed into ATP and polyphosphoric acid (PolyP_{n-2}).

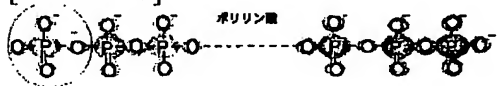
[0017] Under the 1st reaction (one formula) make AMP react with the adenylate kinase and dyad ADP is made to change into the bottom of existence of ATP of a minute amount in this invention, and existence of a polyphosphoric acid compound By repeating the system of reaction of a couple, and the system of reaction of nothing and this couple two or more times, and performing the 2nd reaction (two formulas) which this dyad ADP is made to react with a polyphosphoric acid kinase, and is made to change into dyad ATP and a polyphosphoric acid compound ATP is made to amplify by the exponentiation of 2 according to the count of a reaction.

[0018] Thereby, in the 1st reaction of the system of reaction, ATP and AMP react promptly and are changed into dyad ADP by the adenylate kinase (formula 1). Subsequently, by the polyphosphoric acid kinase, this dyad ADP reacts with polyphosphoric acid, and is changed into dyad ATP and polyphosphoric acid (formula 2). And although it moves to the 2nd reaction of the system of reaction, at this time, said dyad ATP produced in the 1st system of reaction reacts with dyad AMP, and is changed into ADP of four molecules (formula 3). Subsequently, by the polyphosphoric acid kinase, ADP of these four molecules reacts with polyphosphoric acid, and is changed into ATP of four molecules, and polyphosphoric acid (formula 4). Hereafter, ATP increases by being repeated two or more times with the 3rd time of the system of reaction, the 4th time of the system of reaction, and 5th time -- of the system of reaction. ATP of the minute amount added to the 1st time of the system of reaction becomes a trigger, and henceforth, it happens continuously, as long as AMP and polyphosphoric acid exist, it will continue for a long time, and according to the count of a reaction of the system of reaction, ATP will increase this reaction by the exponentiation of 2.

[0019] As for the polyphosphoric acid by which polyphosphoric acid (PolyP) is that with which n phosphoric acids were connected, for example, chemosynthesis was carried out, about 100 phosphoric acids are connected. Moreover, a phosphoric acid with 1000 near things taken out from bacteria is connected.

[0020] For example, the polyphosphoric acid compound (PolyP_n) used by this invention is expressed by the following structure expressions. Here, the range of (PolyP_n) of 10 ≤ n ≤ 1000 is desirable.

[Formula 3]



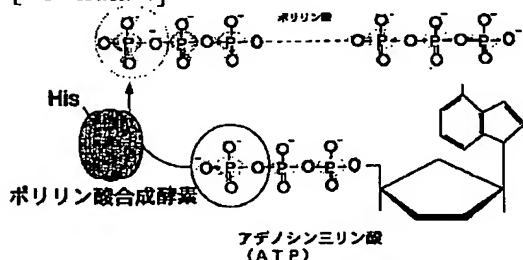
[0021] Said polyphosphoric acid compound is a polyphosphoric acid compound of the bacteria origin, and since at least 10-1000 phosphoric acids (MI3P) are carrying out the polymerization, its reactivity improves.

[0022] If biosynthesized from ATP by the catalysis of polyphosphoric acid synthetic enzyme, the yield of a polyphosphoric acid compound will be improved and it will become possible to generate a polyphosphoric acid compound cheaply. For example, as shown in the following reaction formulae, a

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polyphosphoric acid compound is biosynthesized from ATP.

[Formula 4]



[0023] The biosynthesis of the polyphosphoric acid compound by the above-mentioned reaction formula should just use the manufacture approach of the conventional polyphosphoric acid indicated by JP,5-153993,A etc. With this operation gestalt, metal ions, such as magnesium aiming at deactivation control of polyphosphoric acid synthetic enzyme, ATP, and an enzyme, are made to react by making polyphosphoric acid synthetic enzyme into a catalyst, and a polyphosphoric acid compound is biosynthesized. The polyphosphoric acid synthetic enzyme used for this operation gestalt just biosynthesizes polyphosphoric acid synthetic enzyme. [0024] About the connective magnification reaction of ATP, even if this invention persons used creatine kinase and creatine phosphate instead of polyphosphoric acid and a polyphosphoric acid kinase, they checked that a reaction occurred. That is, if it is the phosphoric-acid compound and enzyme which can change dyad ADP into dyad ATP, ATP will be theoretically considered with making a magnification reaction cause continuously. However, since polyphosphoric acid has the capacity which compounds much ATP by the monad by combining with a polyphosphoric acid kinase, it is advantageous to the magnification reaction of ATP which occurs continuously.

[0025]

[Example 1] In order to compare the conditions to which ATP was made to react by additive-free with the conditions to which added and ATP was made to react, a change of luminescence with time was investigated on the following conditions.

[0026]

① ATP 無添加で反応させた場合

(イ) ポリリン酸キナーゼ緩衝液	10 μ l
(ロ) アデノシン 1 リン酸 (AMP)	7.5 μ l
(ハ) 3 mM ポリリン酸	22.5 μ l
(ニ) ポリリン酸キナーゼ	15 μ l
(ホ) アデニレートキナーゼ	3 μ l
(ヘ) 蒸留水	17 μ l
合計	75 μ l

The above-mentioned (**) - (passing) a sample were mixed, it sampled for every measuring time (5microl), and ATP capacity was measured with the Boehringer Mannheim ATP measurement kit.

[0027]

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②ATP添加で反応させた場合

(イ) ポリリン酸キナーゼ緩衝液	10 μ l
(ロ) アデノシン1リン酸 (AMP)	7.5 μ l
(ハ) 3 mM ポリリン酸	22.5 μ l
(ニ) ポリリン酸キナーゼ	15 μ l
(ホ) アデニル酸キナーゼ	3 μ l
(ヘ) 1.65 μ M ATP	5 μ l
(ト) 蒸留水	12 μ l
合計	75 μ l

** The sample of the above-mentioned (**) - (**) was mixed similarly, it sampled for every measuring time (5microl), and ATP capacity was measured with the Boehringer Mannheim ATP measurement kit. [0028] The result of the above-mentioned ** and the result of ** are shown in drawing 2. When it adds and ATP is made to react from this result (reaction **), the amount of ATP rises rapidly from 30 minutes after a reaction, and a peak is reached after 180-minute progress. On the other hand, when it is made to react by ATP additive-free (reaction **), the amount of ATP does not increase, and while it has been low level, it changes. Therefore, in this invention, it turns out that it became a trigger to have added the minute amount ATP and the connective increment in ATP took place. Therefore, it is possible for ATP of few amounts to also make the amount of ATP increase, to be able to detect it with sufficient sensibility, and it to improve the precision of food evaluation and health inspection. Moreover, ATP is detectable in easy cheap and RUMINO meter.

[0029]

[Effect of the Invention] Under the 1st reaction according to this invention make AMP react with the adenylate kinase and dyad ADP is made to change into the bottom of existence of ATP of a minute amount as mentioned above, and existence of a polyphosphoric acid compound The 2nd reaction which this dyad ADP is made to react with a polyphosphoric acid kinase, and is made to change into dyad ATP and a polyphosphoric acid compound by repeating the system of reaction of a couple, and the system of reaction of nothing and this couple two or more times, and making them react Since ATP is made to amplify by the exponentiation of 2 according to the count of a reaction, ATP of the minute amount added to the 1st time of the system of reaction becomes a trigger, and the increment in ATP takes place continuously henceforth. And the connective increment in ATP will be continued for a long time, as long as AMP and polyphosphoric acid exist, and according to the count of a reaction of the system of reaction, ATP will increase by the exponentiation of 2. Then, if ATP which increased is detected by the bioluminescence, enhancement of the huge quantity of light will take place compared with ATP of the minute amount added to the 1st time of the system of reaction.

[0030] In case polyphosphoric acid and ADP are made to react and ATP is compounded, it becomes possible to make ATP increase continuously. Thereby, while acquiring the enhancing effect of the quantity of light of a bioluminescence, luminescence time amount can be made to maintain.

[0031] Moreover, said polyphosphoric acid compound is a polyphosphoric acid compound generated by chemosynthesis, and is good to use that in which at least 10-100 phosphoric acids carried out the polymerization to the shape of a straight chain. Thereby, since at least 10-100 phosphoric acids (MI3P) are contained in 1 molecule of a polyphosphoric acid compound, continuous conversion to ATP from ADP is performed easily.

[0032] And said polyphosphoric acid compound will be a polyphosphoric acid compound of the bacteria origin, and if that in which at least 10-1000 phosphides carried out the polymerization to the shape of a straight chain is used, conversion to ATP from ADP is performed still more easily, makeup of a phosphoric acid will become little and it will become economical. Moreover, the reaction time of the system of reaction carries out long duration continuation with many phosphoric acids.

[0033] Furthermore, if said polyphosphoric acid compound is biosynthesized from ATP by the catalysis of polyphosphoric acid synthetic enzyme, it will improve the yield of a polyphosphoric acid compound

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and will become possible [generating a polyphosphoric acid compound cheaply]. [0034] If the amount of luminescence which was made to amplify ATP of ultralow volume by the approach of making ATP amplifying continuously, was made to react with luciferase under existence of luciferin and dissolved oxygen, was made to generate AMP and luminescence, and was generated is measured Since the quantity of light equivalent to ATP of the part which increased is obtained, ATP of the ultralow volume which was not able to be detected conventionally can be detected now, for example, it can detect with the brightness of 1000 times or more as compared with an approach without magnification of ATP, and the sensibility and precision of measurement of a bioluminescence improve exceptionally.

[0035] Moreover, since magnification of ATP takes place, by the conventional approach, ATP of the ultralow volume which was not able to be detected is detectable with this invention. Therefore, the microorganism which is not visible at food works etc. is detected, and cleanliness can be inspected or it can apply to measuring the freshness of food, such as meat, a fresh fish, and vegetables. Thus, it applies to the health administration by detection of a minute amount harmful microorganism, and also ATP is produced or it can apply to inspection of a general biochemical reaction which consumes ATP.

Moreover, the application to the scientific criminal investigation which changes to a luminol reaction is also considered by detecting ATP. Moreover, it is applicable to synthetic production of ATP etc.

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DESCRIPTION OF DRAWINGS

[Brief Description of the Drawings]

[Drawing 1] It is drawing which compared the case where added and the case where ATP is made to react by additive-free in the connective magnification reaction of ATP, and ATP were made to react.

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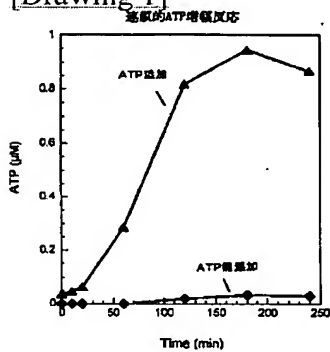
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DRAWINGS

[Drawing 1]



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最終頁に続く

(54) 【発明の名称】 A T P を連鎖的に増幅させる方法及び該方法を利用して極微量の A T P を検査する方法

(57) 【要約】

【課題】新規に A T P を連鎖的に増幅させる方法を発明するとともに、該方法を利用して生物発光の測定の感度と精度を獲得して微量 A T P を検査する方法を提供する。

【解決手段】微量の A T P の存在下に、A M P をアデニレートキナーゼと反応させて2分子の A D P に変換せしめる第1の反応と、ポリリン酸化合物の存在下で、該2分子の A D P をポリリン酸キナーゼと反応させて2分子の A T P とポリリン酸化合物に変換せしめる第2の反応とを一對の反応系となし、該一對の反応系を複数回繰り返して反応させることにより、その反応回数に応じて2のべき乗で A T P を増幅させる。

【特許請求の範囲】

【請求項1】微量のATPの存在下に、AMPをアデニレートキナーゼと反応させて2分子のADPに変換せしめる第1の反応と、ポリリン酸化合物の存在下で、該2分子のADPをポリリン酸キナーゼと反応させて2分子のATPとポリリン酸化合物に変換せしめる第2の反応とを一对の反応系となし、該一对の反応系を複数回繰り返して反応させることにより、その反応回数に応じて2のべき乗でATPを増幅させることを特徴とするATPを連鎖的に増幅させる方法。

【請求項2】前記ポリリン酸化合物は、化学合成により生成されたポリリン酸化合物であって、少なくとも10～100個のリン酸が直鎖状に重合したものをを用いてなる請求項1記載のATPを連鎖的に増幅させる方法。

【請求項3】前記ポリリン酸化合物は、バクテリア由来のポリリン酸化合物であって、少なくとも10～100個のリン酸が直鎖状に重合したものをを用いてなる請求項1記載のATPを連鎖的に増幅させる方法。

【請求項4】前記ポリリン酸化合物は、ポリリン酸合成酵素の触媒作用により、ATPから生合成してなる請求項3記載のATPを連鎖的に増幅させる方法。

【請求項5】請求項1から請求項4のいずれかに記載のATPを連鎖的に増幅させる方法により極微量のATPを増幅させ、ルシフェリン及び溶存酸素の存在下でルシフェラーゼと反応させてAMP及び発光を生成せしめ、生成した発光量を測定することにより極微量のATPを検査する方法。

【発明の詳細な説明】

【0001】

【発明が属する技術分野】本発明は、動植物の代謝・生

合成系において生成されるATPの量を、連鎖的に増加させる方法であり、該方法を利用して生物発光を検出することで、従来検出できなかった極微量のATPが検出できるために、食品工場などで目に見えない微生物を検出して清浄度を検査したり、食肉、鮮魚、野菜など食物の鮮度を測定することに応用できるものである。

【0002】

【従来の技術】ATPは生きた生物の指標である。従って、微生物由来のATPを生物発光に供することによって、光を指標にした微生物の衛生検査が行われている。

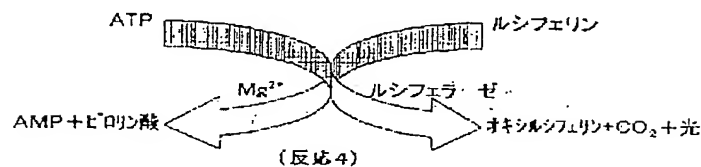
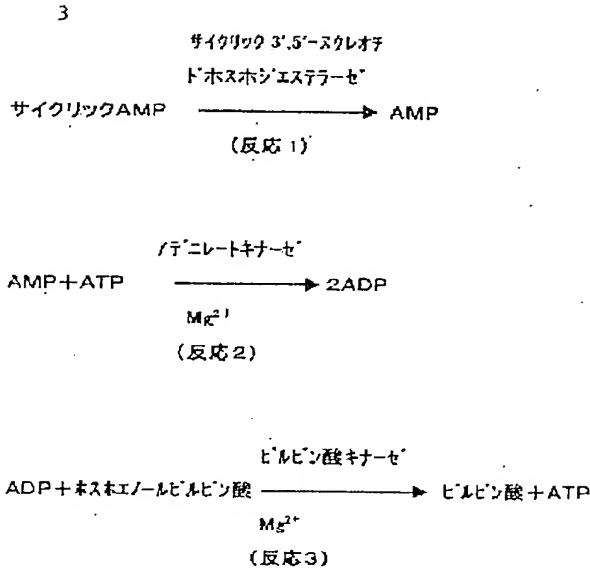
（例えば、特公平6-34757,登録第1911659号）しかし、微量の微生物の場合にはATPに由来する生物発光が十分に行われない。

【0003】そこで、特開平8-47399号公報には、「ルシフェラーゼにより発生する生物発光を測定する方法において、ポリリン酸化合物又はその塩、及びスルフィドリル化合物の共存下で生物発光反応を行う」ことを特徴とする技術が開示されている。これにより、生物発光の増強という新規な効果を得られ、一般的なルミノメータの使用により生物発光反応の測定が可能で、普及率が高まるという効果がある。

【0004】しかしながら、上記特開平8-47399に開示される方法は、生物発光の安定の技術であって、ATP量を増加させる技術ではなく、ATPが消費されるに従い、経時的に発光が減衰する欠点を有する。

【0005】また、特開平9-234099号には、以下の反応式で示される生物発光法によるサイクリックAMPの定量法が開示されている。

【化1】



【0006】この方法は、サイクリックAMPをサイクリック3',5'-ヌクレオチドホスホジエステラーゼで加水分解して反応系にAMPを生成せしめる(反応1)と、マグネシウムイオン、微量のATPの存在下に、該AMPをアデニレートキナーゼと反応させてADPに変換せしめる(反応2)と、マグネシウムイオン、ホスホエノールピルビン酸の存在下で、該ADPをピルビン酸キナーゼと反応させて、ATP及びピルビン酸に変換せしめる(反応3)と、ルシフェリン、マグネシウムイオン(又は他の金属イオン)及び溶存酸素の存在下で、ATPをルシフェラーゼと反応させ発光を生成せしめる(反応4)と、(反応4)で生成した発光量を測定することによりサイクリックAMPを定量する方法(METHODS IN ENZYMOLOGY 38,62-65;1974)を特徴としている。

【0007】しかしながら、この方法は、サイクリックAMPから変換されたAMPを、ADPに変換し、さらに該ADPをホスホエノールピルビン酸の存在下でピルビン酸キナーゼの反応によりATPとしてATPのリサイクルが行われるが、ATP量が増加しているわけではない。

【0008】

【発明が解決しようとする課題】本発明は、上記従来技術とは全く異なる観点でATP量を連鎖的に増加させる方法を発明するとともに、該方法を利用して生物発光を

検出することで、従来検出できなかった極微量のATPを検出する方法を提供することを技術的課題とする。

【0009】

【課題を解決するための手段】上記課題を解決するため請求項1の発明は、微量のATPの存在下に、AMPをアデニレートキナーゼと反応させて2分子のADPに変換せしめる第1の反応と、ポリリン酸化合物の存在下で、該2分子のADPをポリリン酸キナーゼと反応させて2分子のATPとポリリン酸化合物に変換せしめる第2の反応とを一対の反応系となし、該一対の反応系を複数回繰り返して反応させることにより、その反応回数に応じて2のべき乗でATPを増幅させる、という技術的手段を講じた。

【0010】反応系の1回目の反応において、アデニレートキナーゼによってATPとAMPとが直ちに反応し、2分子のADPに変換される(第1の反応)。次いで、該2分子のADPはポリリン酸キナーゼによってポリリン酸と反応し、2分子のATPとポリリン酸に変換される(第2の反応)。そして、反応系の2回目の反応に移るが、このとき、反応系1回目で生じた前記2分子のATPが2分子のAMPと反応し、4分子のADPに変換される(第1の反応)。次いで、該4分子のADPはポリリン酸キナーゼによってポリリン酸と反応し、4分子のATPとポリリン酸に変換される(第2の反応)。以下、反応系の3回目、反応系の4回目、反応系

の5回目…と複数回繰り返されることによりATPが増加する。この反応は、反応系の1回目に添加する微量のATPが引き金になり、以降、連鎖的に起こり、AMPとポリリン酸が存在する限り長時間継続し、反応系の反応回数に応じて2のべき乗でATPが増加することになる。

【0011】また、前記ポリリン酸化合物は、化学合成により生成されたポリリン酸化合物であって、少なくとも10～100個のリン酸が直鎖状に重合したものをを用いるとよい。ポリリン酸化合物(PolyP_n)とはn個のリン酸がつながったもので、請求項2の発明では、ポリリン酸化合物の1分子中にリン酸(Mⁱ, P)が少なくとも10～100個つながっているので、請求項1の第2の反応におけるADPからATPへの変換が容易に行われ、ATPを再生する際のリン酸の補給が少量となり経済的になる。また、多数のリン酸により反応系の反応時間が継続する。

【0012】さらに、前記ポリリン酸化合物は、バクテリア由来のポリリン酸化合物であって、少なくとも10～1000個のリン酸が直鎖状に重合したものをを用いるとよい。請求項3の発明では、ポリリン酸化合物の1分*

*子中にリン酸(Mⁱ, P)が少なくとも10～1000個つながっているため、ADPからATPへの変換がさらに容易に行われ、リン酸の補給が少量となり経済的となる。また、多数のリン酸により反応系の反応時間が長時間継続する。

【0013】そして、前記ポリリン酸化合物は、ポリリン酸合成酵素の触媒作用により、ATPから生合成すれば、ポリリン酸化合物の収率を向上して、ポリリン酸化合物を安価に生成することが可能となる。

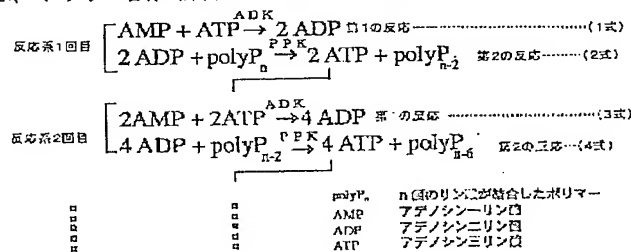
【0014】ATPを連鎖的に増幅させる方法により極微量のATPを増幅させ、ルシフェリン及び溶存酸素の存在下でルシフェラーゼと反応させてAMP及び発光を生成せしめ、生成した発光量を測定すると、増加した分のATPに相当する光量が得られるため、従来検出できなかった極微量のATPが検出できるようになる。原理的には、最初の反応系に存在する一分子のATPをも検出できるものである。

【0015】

【発明の実施の形態】本発明の実施の形態を説明する。

以下に本発明の理論的な反応式を示す。

【化2】



【0016】反応の触媒となるアデニレートキナーゼ(ADK)は、アデノシン一リン酸(AMP)とアデノシン三リン酸(ATP)とを反応させたとき、2分子のアデノシン二リン酸(ADP)を生じさせる酵素である。また、ポリリン酸キナーゼ(PPK)は、ADPとポリリン酸(PolyP_n)とを反応させ、ATPとポリリン酸(PolyP_{n-2})とに変換する酵素である。

【0017】本発明では、微量のATPの存在下に、AMPをアデニレートキナーゼと反応させて2分子のADPに変換せしめる第1の反応(1式)と、ポリリン酸化合物の存在下で、該2分子のADPをポリリン酸キナーゼと反応させて2分子のATPとポリリン酸化合物に変換せしめる第2の反応(2式)とを一对の反応系となし、該一对の反応系を複数回繰り返して行うことにより、その反応回数に応じて2のべき乗でATPを増幅させるのである。

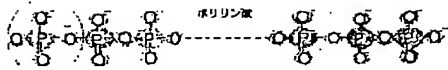
【0018】これにより、反応系の1回目の反応において、アデニレートキナーゼによってATPとAMPとが直ちに反応し、2分子のADPに変換される(式1)。次いで、該2分子のADPはポリリン酸キナーゼによってポリリン酸と反応し、2分子のATPとポリリン酸に

30 変換される(式2)。そして、反応系の2回目の反応に移るが、このとき、反応系1回目で生じた前記2分子のATPが2分子のAMPと反応し、4分子のADPに変換される(式3)。次いで、該4分子のADPはポリリン酸キナーゼによってポリリン酸と反応し、4分子のATPとポリリン酸に変換される(式4)。以下、反応系の3回目、反応系の4回目、反応系の5回目…と複数回繰り返されることによりATPが増加する。この反応は、反応系の1回目に添加する微量のATPが引き金になり、以降、連鎖的に起こり、AMPとポリリン酸が存在する限り長時間継続し、反応系の反応回数に応じて2のべき乗でATPが増加することになる。

【0019】ポリリン酸(PolyP)とはn個のリン酸がつながったもので、例えば、化学合成されたポリリン酸は100個ほどのリン酸がつながったものである。また、バクテリアから取り出したものは1000個近いリン酸がつながったものである。

【0020】例えば、本発明で用いられるポリリン酸化合物(PolyP_n)は、以下の構造式によって表される。ここで、(PolyP_n)は10 ≤ n ≤ 1000の範囲が好ましい。

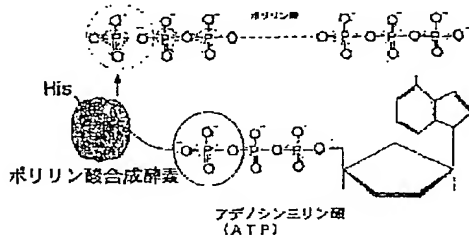
【化3】



【0021】前記ポリリン酸化合物は、バクテリア由来のポリリン酸化合物であって、少なくとも10～100個のリン酸(M⁺、P)が重合しているので反応性が向上する。

【0022】ポリリン酸合成酵素の触媒作用により、ATPから生合成すれば、ポリリン酸化合物の収率を向上して、ポリリン酸化合物を安価に生成することが可能となる。例えば、以下の反応式に示すようにポリリン酸化合物を、ATPから生合成する。

【化4】



【0023】上記反応式によるポリリン酸化合物の生合成は、例えば、特開平5-153993号公報などに開示された従来のポリリン酸の製造方法を利用すればよい。本実施形態では、ポリリン酸合成酵素を触媒として、ポリリン酸合成酵素とATPと酵素の失活抑制を目的としたマグネシウムなどの金属イオンを反応させ、ポリリン酸化合物を生合成する。本実施形態に使用されるポリリン酸合成酵素はポリリン酸合成酵素を生合成し得るものであればよい。

【0024】本発明者らは、ATPの連鎖的増幅反応について、ポリリン酸及びポリリン酸キナーゼの代わりにクレアチンキナーゼ及びクレアチンリン酸を使用しても反応が起こることを確認した。すなわち、2分子のADPを2分子のATPに変換することのできるリン酸化合物と酵素であれば、原理的にはATPを連鎖的に増幅反応を起こさせると考えられる。しかし、ポリリン酸はポリリン酸キナーゼと組み合わせることにより、一分子で多数のATPを合成する能力があるので、連続して起こるATPの増幅反応に有利である。

【0025】

【実施例1】ATPを無添加で反応させた条件と、ATPを添加して反応させた条件とを比較するため、以下の条件で発光の経時的変化を調べた。

【0026】

①ATP無添加で反応させた場合

(イ) ポリリン酸キナーゼ緩衝液	10μl
(ロ) アデノシン1リン酸(AMP)	7.5μl
(ハ) 3mMポリリン酸	22.5μl
(ニ) ポリリン酸キナーゼ	15μl
(ホ) アデニレートキナーゼ	3μl
(ヘ) 蒸留水	17μl
合計	75μl

上記(イ)～(ヘ)の試料を混合し、測定時間ごとにサンプリング(5μl)を行い、ペーリンガーマンハイム社製のATP測定キットにより、ATP容量を測定した。

【0027】

②ATP添加で反応させた場合

(イ) ポリリン酸キナーゼ緩衝液	10μl
(ロ) アデノシン1リン酸(AMP)	7.5μl
(ハ) 3mM ポリリン酸	22.5μl
(ニ) ポリリン酸キナーゼ	15μl
(ホ) アデニル酸キナーゼ	3μl
(ヘ) 1.65μM ATP	5μl
(ト) 蒸留水	12μl
合計	75μl

①と同様に上記(イ)～(ト)の試料を混合し、測定時間ごとにサンプリング(5μl)を行い、ペーリンガーマンハイム社製のATP測定キットにより、ATP容量を測定した。

【0028】上記①の結果及び②の結果を図2に示す。この結果から、ATPを添加して反応させた場合(反応②)、反応後30分からATP量が急激に上昇し、180分経過後にはピークに達する。これに対し、ATP無添加で反応させた場合(反応①)、ATP量が増加することではなく、低水準のまま推移する。従って、本発明では、微量ATPを添加したことが引き金となって、ATPの連鎖的増加が起こったことが分かる。従って、わずかな量のATPでもATP量を増加させて感度よく検出することができ、食品検査、衛生検査の精度を向上することが可能である。また、安価でかつ簡単なルミノメータによりATPを検出することができる。

【0029】

【発明の効果】以上のように本発明によれば、微量のATPの存在下に、AMPをアデニレートキナーゼと反応させて2分子のADPに変換せしめる第1の反応と、ポリリン酸化合物の存在下で、該2分子のADPをポリリン酸キナーゼと反応させて2分子のATPとポリリン酸化合物に変換せしめる第2の反応とを一对の反応系となし、該一对の反応系を複数回繰り返して反応させることにより、その反応回数に応じて2のべき乗でATPを増幅させるので、反応系の1回目には添加する微量のATPが引き金になり、以降、連鎖的にATPの増加が起こる。そして、ATPの連鎖的増加は、AMPとポリリン

酸が存在する限り長時間継続し、反応系の反応回数に応じて2のべき乗でATPが増加することになる。この後、増加したATPを生物発光で検出すると、反応系の1回目に添加する微量のATPに比べて、膨大な光量の増強が起こる。

【0030】ポリリン酸とADPとを反応させてATPを合成する際、連鎖的にATPを増加させることが可能となる。これにより、生物発光の光量の増強効果を得るとともに、発光時間を持続させることのできる。

【0031】また、前記ポリリン酸化合物は、化学合成により生成されたポリリン酸化合物であって、少なくとも10～100個のリン酸が直鎖状に重合したものをを用いるとよい。これにより、ポリリン酸化合物の1分子中にリン酸(M⁺、P)が少なくとも10～100個含まれているので、ADPからATPへの連続的な変換が容易に行われる。

【0032】そして、前記ポリリン酸化合物は、バクテリア由来のポリリン酸化合物であって、少なくとも10～1000個のリン酸が直鎖状に重合したものをを用いると、ADPからATPへの変換がさらに容易に行われ、リン酸の補給が少量となり経済的となる。また、多数のリン酸により反応系の反応時間が長時間継続する。

【0033】さらに、前記ポリリン酸化合物は、ポリリン酸合成酵素の触媒作用により、ATPから生合成すれば、ポリリン酸化合物の収率を向上して、ポリリン酸化*

* 化合物を安価に生成することが可能となる。

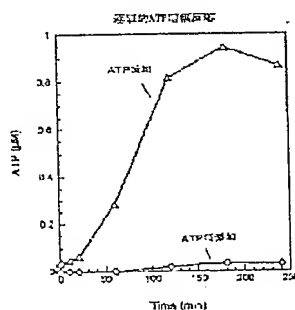
【0034】ATPを連鎖的に増幅させる方法により極微量のATPを増幅させ、ルシフェリン及び溶存酸素の存在下でルシフェラーゼと反応させてAMP及び発光を生成せしめ、生成した発光量を測定すると、増加した分のATPに相当する光量が得られるため、従来検出できなかった極微量のATPが検出できるようになり、例えば、ATPの増幅を伴わない方法と比較して1000倍以上の明るさで検出できるようになり、生物発光の測定の感度と精度が格別向上する。

【0035】また、本発明ではATPの増幅が起こるため、従来の方法では検出できなかった極微量のATPを検出できる。そのために、食品工場などで目に見えない微生物を検出して清浄度を検査したり、食肉、鮮魚、野菜など食物の鮮度を測定することに応用できるものである。このように、微量有害微生物の検出による衛生管理に応用する他、ATPを生ずる、あるいは、ATPを消費するような一般的な生化学反応の検査にも応用できる。また、ATPを検出することによって、ルミノール反応にかかわる科学捜査などへの応用も考えられる。また、ATPの合成生産などにも応用することができる。

【図面の簡単な説明】

【図1】ATPの連鎖的増幅反応においてATPを無添加で反応させた場合とATPを添加して反応させた場合とを比較した図である。

【図1】



フロントページの続き

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